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Abstract

The temporal coordination of anaphase, cytokinesis and mitotic exit is essential for the production of viable daughter cells, and mutations that affect the proper timing of these events result in genomic instability, a hallmark of cancer. In yeast, a signaling pathway has been identified, called the Mitotic Exit Network, which coordinates mitotic exit and cytokinesis with the end of anaphase. The identification and characterization of such a pathway in human cells is necessary to further our understanding of how normal cell division is regulated and will highlight possible mechanisms of genomic instability in tumor cells. In order to discover those genes that are involved specifically in animal cell division and are not conserved in yeast, we are taking advantage of the nematode, *C. elegans*. Using the yeast two-hybrid system, we have mapped the physical interactions of many *C. elegans* genes believed to be potential mitotic regulators. The goal was to generate a large protein interaction map, which in combination with phenotypic data will help to elucidate the biochemical pathways involved in cooridinating mitotic events in animal cells.

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Introduction

The temporal coordination of anaphase, cytokinesis and mitotic exit is essential for the production of viable daughter cells, and mutations that affect the proper timing of these events result in genomic instability, a hallmark of cancer. In yeast, a signaling pathway has been identified, called the Mitotic Exit Network, which coordinates mitotic exit and cytokinesis with the end of anaphase. Homologues of three of these signaling components have been identified in humans suggesting that human cells regulate mitosis in a similar fashion; however, a clear mitotic exit network has yet to be revealed. The identification and characterization of such a pathway in human cells will further our understanding of how normal cell division is regulated and will highlight possible mechanisms of genomic instability in tumor cells. In order to discover those genes that are involved specifically in animal cell division and are not conserved in yeast, we are taking advantage of the nematode, *C. elegans*. *C. elegans* is a multi-cellular complex metazoan whose genes are more homologous to humans than are those of yeast. This system should allow for the rapid functional analysis of large numbers of candidate genes that can then be used to ascertain their human counterparts by sequence comparison. Using the yeast two-hybrid system we have built a protein interaction map for hundreds of candidate *C. elegans* genes that are potentially involved in mitotic temporal control, based on phenotypic data as well as homology to yeast genes of known function. The goal was to generate a large protein interaction map, which in combination with phenotypic data will help to elucidate the biochemical pathways involved in coordinating mitotic events in animal cells. By recognizing the components of these putative pathways novel targets for anti-cancer therapies may be discovered. During the first twelve months of this project we were able to generate a preliminary interaction map based on the raw two-hybrid data. This report describes our efforts over the last year to refine that data set and combine it with phenotypic data in order to determine the biological relevance of these interactions. Unfortunately, these efforts culminated in the conclusion that the two-hybrid data do not reflect biological interactions and have not lead to the discovery of a novel biochemical pathway, which was the intention. However, recent work from others in the field has compelled us to approach this problem from a different direction, as is described in this report and in the appended statement-of-work.

Body

1. **Refine protein-interaction map for mitosis in *C. elegans*:** We started with a set of 324 *C. elegans* genes, which were determined based on homology to yeast genes and on RNAi phenotypes to be likely to be involved in mitosis. As described previously, we performed a yeast two-hybrid matrix experiment, in which each gene product was directly tested for interaction with every other gene product in the collection. We also performed full genome two-hybrid screens with selected bait constructs. The raw data were collected and added to additional data from our collaborator, Dr. Marc Vidal. Using a computer program, Osprey, we constructed an interaction map of all the yeast two-hybrid data. This map contained 293 proteins, making 101 connections. In order to refine the data set, we then removed those hits that we felt were unlikely to be biologically relevant for mitosis. For example, mitochondrial proteins and cysteine proteases were common hits and were determined to be artifacts. Exceptions were made for those genes whose reported RNAi phenotypes implicated them in some aspect of cell division. We also retained hits based on the predicted domain structure of the product (kinases, G-proteins, microtubule-binding, etc). This left us with a much more manageable data set, comprising 40 genes making 56 connections. Finally, we removed all those genes whose cell cycle phenoypes were likely to be caused by indirect effects. For example, many ribosomal genes were taken out with the assumption that they could not contribute to the pathways of interest. When we did this, a core interaction set of only 12 genes could be seen centering on the gene, zyg-8. This small network seemed to be the most likely place to start looking for the mitotic exit network (figure 1).

2. **Record RNAi phenotypes for the most interesting candidates:** The RNAi phenotypes for the genes in our screen had largely been characterized and recorded in WormBase. As most of these data came from large-scale studies, we repeated the RNAi experiments to confirm the phenotypes of the zyg-8 network genes. These experiments were carried out by the feeding method of RNAi, where worms are fed *E. coli* that express the RNAi constructs off of plasmids. The plasmids were constructed using the Gateway recombinational cloning system that was used to construct the yeast two-hybrid constructs. In all cases, we were able to confirm the previously recorded phenotypes. If we had been able to confirm any of the protein-protein interactions predicted in our yeast two-hybrid results, the next step would

have been to do epistatic analyses to order the pathway. These experiments would have been done by feeding the worms two or more RNAi constructs simultaneously and looking for the epistatic phenotypes. However, we were unable to confirm any of the two-hybrid data (see below) and did not carry on with the epistasis analysis.

3. **Confirm yeast two-hybrid data using alternative assays.** The yeast two-hybrid matrix experiment produced a much lower signal-to-noise ratio than would be considered acceptable. However, there were known interactions that were detected, and so, it is likely that at least some of the data represented biologically relevant interactions. However, due to the high background, it was of particular importance that the novel interactions be confirmed using alternative protein-protein interaction assays. The first that was attempted was an *in vitro* binding assay using *in vitro* transcribed proteins. The yeast two-hybrid constructs contain the appropriate elements to allow for *in vitro* transcription/translation of the genes of interest. The plan was to make S^{35}-labeled protein from each gene in a given interaction pair, mix the two proteins together, and then using an antibody to the DNA binding portion of the bait protein, detect the interaction by co-immunoprecipitation. What we discovered was that many of the genes of interest could not be expressed off of the yeast two-hybrid plamids using the in vitro translation kit. In addition, we were unable to detect the expression of many of these constructs in the yeast two-hybrid strain by western blot. The final attempt at confirming these interactions was to transfer the genes of interest into mammalian expression vectors, co-transfect interacting pairs into NIH 3T3 cells, and perform co-IPs to detect interactions. We attempted this assay exhaustively, but we were unable to confirm any of the two-hybrid data for those interactions that were most interesting.

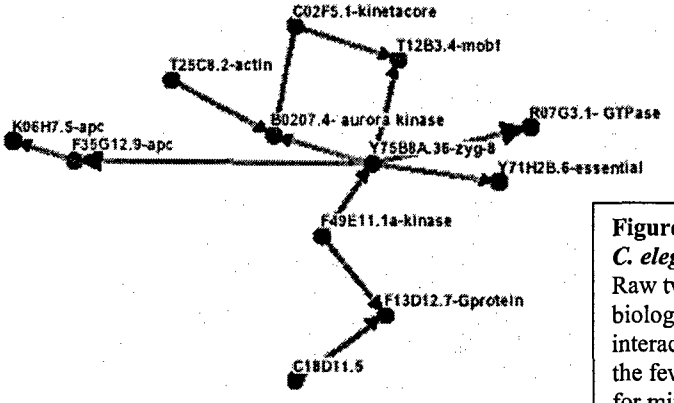

Figure 1: Core Two-Hybrid Interaction Map for Mitosis in *C. elegans*
Raw two-hybrid data was compiled and analyzed for potential biological relevance according to what was known about each interacting partner and common sense. This map represents only the few interactions which are considered to be the most interesting for mitosis at this time.

4. **Begin the characterization of a conserved gene with an interesting phenotype.** Our failure to confirm the yeast two-hybrid data by other methods led us to abandon that approach. We've turned our attentions to another aspect of chromosome segregation and have begun the characterization of a yeast gene, *RAT1/XRN2*. The null phenotype of this gene suggests to us that it might be important in connecting anaphase and mitotic exit. *RAT1* is an essential gene that encodes an exonuclease. Its' role in transcription termination has recently been characterized in both yeast and human cells. However, this is not the essential function. The gene is exceptionally well conserved from yeast to humans, and it has been shown that the mouse homologue, Dhm1, is able to compliment the *pombe* mutant, dhp1. In budding and fission yeast, *rat1* null strains arrest in anaphase of their first cell division with chromatid bridges (figure 2). Often these cells divide their cytoplasms, despite the fact that their chromosomes have not fully divided, leading to a classic "cut" phenotype. This phenotype suggests that these mutants have uncoupled anaphase and mitotic exit regulation.

The 5'-end of telomeres needs to be resected at the end of every S-phase in order to become functional. The phenotypes of less severe mutants of yeast *RAT1* suggests that the product of this gene may catalyze strand resection of telomeres. In addition, these mutants have a delayed cell cycle, suggesting that the function of Rat1p is somehow linked to cell cycle progression. At first, it was not clear how an exonuclease suspected of having a role in telomere processing could affect anaphase and mitotic exit. However, several papers have described a defective anaphase in cells with altered telomeres (ref 1 and 2). This observation has been made in yeast, ciliates, and mammalian cells, but it was work from the Amon lab (MIT) that has really shed light on the possible connection between telomeres and mitotic exit. In their 2004 paper (ref 3), they describe the activation of the FEAR network by late segregating chromosome regions such as nucleoli and telomeres. A reasonable model is that because the telomeric regions of chromosomes are the last to segregate, their separation leads to the activation of the FEAR pathway, which directly activates the MEN. This mechanism of temporal regulation would ensure that mitotic exit always closely follows the end of anaphase, but never becomes activated early. Whatever the mechanism, it seems clear that normal telomere structure is essential for proper chromosome segregation and genomic stability. Therefore, if hRAT1 is required for telomere function, it will potentially provide a target for cancer therapy. Depletion of Rat1p function, either through delivery of a dominant negative allele, or by small molecule inhibitor, is likely to lead to rapid cell death in the first cell cycle of dividing cells. The severity of this therapy would only be enhanced in cells that lack proper checkpoint pathways. Because the other functions of Rat1p seem not to be essential for cell viability, this putative therapy would only affect dividing cells, leaving the majority of healthy somatic cells unaffected.

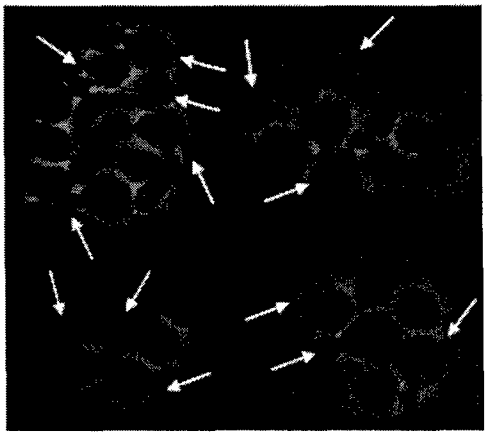

Figure 2. *S. cerevisiae* 4hr after Rat1p depletion (arrows indicate mitotic cells).
Cells expressing a conditional allele of *RAT1* were depleted of Rat1p, and then fixed and stained with DAPI to visualize the DNA. Over the first few hours, these cells begin to accumulate in mitosis with their chromosomes stretched across the bud neck. None of these cells survive the first cell division in the absence of Rat1p.

Key Research Accomplishments

- Completed data collection for yeast two-hybrid screens.
- Refined raw data and arrived at a core set of potentially interesting interactions.
- Generated RNAi constructs for core set of genes and performed RNAi experiments.
- Generated mammalian expression constructed for core set of genes.
- Determined that the yeast two-hybrid data is not reproducible by other methods.
- Began the characterization of RAT1, a yeast gene that may influence the temporal regulation of mitosis.

Reportable Outcomes

None yet, but expecting to have a manuscript in preparation by the end of the calendar year.

Conclusions/Discussion

Although many interactions among the collection of candidate genes have been recorded, none could be determined to be biologically relevant for mitosis in *C. elegans*. One serious concern is that very few of the interactions observed include genes that are currently known to be involved in late mitotic events, either through RNAi phenotypes or by sequence homology to yeast genes. It's possible that proteins interactions that function in mitosis are not readily observed in the yeast two-hybrid system. One possibility is that these interactions are regulated by protein modifications that cannot take place in yeast cells. There are many other potential problems that could have rendered this approach unfeasible. It was particularly alarming that so many constructs could not be expressed using *in vitro* transcription/translation. It was the inability to reproduce these interactions by co-IP from mammalian cell extracts that eventually lead us to abandon this project.
We have had much more luck with a second project, the characterization of an essential gene, RAT1. Although, up until now all of the work has been done with yeast, the high level of conservation of the gene makes it likely that we will observe similar phenotypes for hRAT1.

References

1. **Dynek and Smith (2004) *Science* 304 : 97**
2. **Kirk et. al. (1997) *Science* 275: 1478**
3. **D'Amours D, Stegmeier F, Amon A. (2004) *Cell* 117(4):455-69**

Statement of Work

Task 1. To create the mitosis interactome (Months 1-12)

a. Complete the transfer of mitosis ORFs from ORFeome entry clones to two-hybrid destination vectors (Month 1).
b. Generate yeast strains to be used in the two-hybrid matrix experiment (Month 2)
c. Perform two-hybrid experiments by mating each bait strain to the full array of prey strains and record positive interactions (Months 3-11).
d. Perform two-hybrid screens of worm embryo cDNA library using the most promising candidate genes as baits and record positive interactions (Months 3-11).
e. Analyze raw interaction data and draw a representative interaction map (Month12).

Task 2. To assign mitotic functions to candidate genes (Months 13-18)

a. Make RNAi plasmids for candidate worm MEN genes by subcloning and introduce into RNAi *E. coli* strain (Months 13-14).
b. Perform RNAi experiments against individual genes and record phenotypes (Months 14-16).
c. Perform RNAi experiments against combinations of genes to assess epastatic relationships (Months 17-18).

Task 3. To identify human homologues of worm MEN genes and characterize their mitotic functions (Months 19-36)

a. Identify closest human homologues by BLAST search (Month 19).
b. Design and construct shRNA reagents and cell lines (Months 20-24).
c. Study phenotypes in shRNA cells by microscopy and flow cytometry; record affect on mitosis (Months 25-26)
d. Create GFP-fusion constructs to be used in localization studies (Month 27).
e. Perform localization studies in synchronized cell populations (Month 28).
f. Perform epistasis analysis by observing the localization of certain MEN proteins in strains that have shRNAs to other MEN proteins, in order to establish the order of the pathway (Months 29-30).
g. Perform biochemical analysis on proteins of particular interest (Months 31-36).

REVISED STATEMENT OF WORK: MONTHS 25-36

Task 1: Characterize mitotic function of RAT1 in mammalian cells (months 25-29).

- siRNA in multiple human cell lines, transformed and non-transformed.
- Metaphase spreads of chromosomes after siRNA to detect fusions and translocations.
- Cell cycle progression in siRNA treated cells
- Localization of Rat1 protein using indirect immunofluoresence.
- Monitor activation of both checkpoints by immunofluoresence and western blot.
- Monitor localization of hCdc14 in cells treated with RAT1 siRNA.

Task 2: Characterize role of RAT1 at telomeres in mammalian cells (months 30-36).

- Chromatin IP to detect localization at telomeres.
- Detect ssDNA formation at telomeres after hRAT1 siRNA treatment
- Create dominant negative alleles of hRAT1 and assay affects on telomere length regulation and frequency of ligase IV-dependent telomere fusions.
- Perform in vitro exonuclease assays to measure Rat1 activity on telomere DNA.